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Disruption of Cell Adhesion in *Xenopus* Embryos by Pagliaccio, an Eph-Class Receptor Tyrosine Kinase

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Pagliaccio (Pag) is a receptor tyrosine kinase of the Eph family that is expressed in *Xenopus* embryos in a diverse set of localized tissues. Pag is the *Xenopus* homolog of Hek-8 (human), Sek-1 (mouse), cek8 (chicken), and RTK-1 (zebrafish). We have investigated the function of this protein by injecting RNA encoding an epidermal growth factor receptor–Pag chimera into early *Xenopus* embryos. Activation of the chimeric receptor results in a kinase-dependent loss of cell–cell adhesion. This dissociation can be reversed by co-injection of RNA encoding C-cadherin, suggesting that one or more cadherins could be the functional targets for Pag activity.

INTRODUCTION

The Eph class of receptor tyrosine kinases (RTKs) is a large family (Tuzi and Gulick, 1994), many of which exhibit highly localized expression patterns in vertebrate embryos, especially the central nervous system. Several ligands have recently been identified for these RTKs (Bartley *et al.*, 1994; Beckmann *et al.*, 1994; Cheng and Flanagan, 1994; Davis *et al.*, 1994; Shao *et al.*, 1994; Drescher *et al.*, 1995; Kozlosky *et al.*, 1995; Winslow *et al.*, 1995). All of these ligands are associated with the cell surface through either transmembrane regions or glycosyl phosphatidylinositol linkages, and their activity appears to be dependent upon this association (Davis *et al.*, 1994). Several of these ligands exhibit discrete expression patterns, overlapping with regions expressing Eph-class RTKs (Cheng and Flanagan, 1994, Cheng *et al.*, 1995). These properties have led to the suggestion that Eph-class receptor/ligand interactions could play important roles in patterning of the nervous system and other tissues. The recent identification of two ligands, AL-1 (Winslow *et al.*, 1995) and RAGS (Drescher *et al.*, 1995), which appear capable of modulating axon fasciculation and retinotectal axon targeting, supports this idea. Furthermore, experimental disruption of Eph-class RTKs RTK-1 and XSek-1 in zebrafish and *Xenopus*, respectively, has been shown to result in disruption of boundaries between rhombomeres (Xu *et al.*, 1995).

Pagliaccio (Pag), a gene cloned from *Xenopus* visceral arch neural crest (Winning and Sargent, 1994; Scales *et al.*, 1995), encodes a member of the Eph family of RTKs which is probably the homolog of the murine Sek-1 (Gilardi-Hebenstreit *et al.*, 1992), zebrafish RTK-1 (Xu *et al.*, 1994; 1995), chick cek8 (Sajjadi and Pasquale, 1993), and human Hek8 (Fox *et al.*, 1995). A pseudo-allelic *Xenopus* gene, called XSek-1, has also been recently reported (Xu *et al.*, 1995). Pag is expressed in a specific subset of tissues in the early embryo: involuting mesoderm during gastrulation, forebrain beginning at neurula stage, rhombomeres r3 and r5 of the hindbrain, cranial neural crest of visceral arch 3, and transiently in dorsal otic vesicle and developing pronephros (Winning and Sargent, 1994).

The Eph class is quite complex, and there is a correspondingly large number of ligands, at least some of which can bind and activate multiple receptors (Cheng and Flanagan, 1994; Brambilla *et al.*, 1995). This potential for promiscuity in ligand–receptor interactions could make it difficult to unambiguously interpret experiments in which endogenous ligand or receptor levels are manipulated. To avoid this problem, we have used a chimeric receptor comprising the ligand-binding domain of human epidermal growth factor receptor (EGFR) and the catalytic and membrane-spanning regions of Pag. This approach has been successfully employed in the analysis of other RTKs, including another Eph-class gene, Elk (Lhotàk and Pawson, 1993).

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In this study, we demonstrate that expression of activated chimeric receptor by microinjection of RNA into fertilized frog eggs caused a profound disruption of cell adhesion. This required Pag kinase activity and could be rescued by co-injection of a dominant negative form of the chimeric receptor. Such dissociation could also be prevented or reversed by co-injection of RNA encoding C-cadherin. Based on these observations, we hypothesize that Pag could act in embryonic tissues to modulate cell adhesion, possibly by regulating cadherin function. We discuss how this might relate to the observed functions for Pag as well as other Eph-class receptors.

MATERIALS AND METHODS

Construction of the Chimeric Receptor Clone

The transmembrane and intracellular regions of Pag were isolated on a *Cla*I–*Mlu*I fragment by PCR amplification with Vent polymerase (New England Biolabs Inc.), using the following primers: *Cla* primer, 5′-CCCATCGATACTTTTGGTGTCCGTGGCT-3′ and *Mlu*/reverse primer, 5′-CCCACGCGTCTCGAGTCATCA-AACAGGAACCATTCT-3′. The DNA product was digested with *Cla*I and *Mlu*I, purified by agarose gel electrophoresis, and ligated to similarly digested vector DNA. The vector, pLTR-5M, was generously provided by Paolo Di Fiore. It contains the protein coding sequence of human epidermal growth factor receptor (EGFR) in which a unique *Cla*I site has been engineered just upstream from the EGFR transmembrane domain, and a unique *Mlu*I site downstream from the EGFR translational termination codon (Lonardo *et al.*, 1990). Due to a *Cla*I site located in the cytoplasmic domain of Pag, it was necessary to insert a small *Cla*I–*Cla*I fragment separately. The resulting clone, called EPP, comprises EGFR from the amino terminus to the serine residue at position 621 (Ullrich *et al.*, 1984) and Pag from the leucine residue at position 549 to the carboxy terminus. All but the first amino acid of the transmembrane domain is thus derived from Pag. The DNA sequence was verified for all positions generated by PCR.

An *Xho*I fragment consisting of the entire EPP open reading frame was then inserted into the *Xho*I site of pSP64-S (a gift from S. Sokol), producing a clone named pEPP-64S. A mutated form of the chimeric receptor lacking kinase activity was constructed by introducing two base changes in the ATP-binding region, which substitutes alanine for lysine at residue number 753 of EPP. The base substitutions were accomplished by performing two parallel PCR amplifications, using Vent polymerase, of adjoining segments of the Pag intracellular domain. The primers used for the first reaction were: upstream primer, 5′-TGGACCCATTTACATATG-3′ and downstream (mutagenic) primer, 5′-CTTTCAGGGTCGCAA-TTGCAACAT-3′. The primers for the second reaction were: upstream (mutagenic) primer, 5′-ATGTTGCAATTGCGACCCTGA-AAG-3′ and downstream primer, 5′-TCCCACAAGCTGTATTAC-3′. Products from these reactions were mixed (after removal of primers), annealed, and amplified using the upstream primer from reaction one and the downstream primer of reaction two. The resulting 385-bp fragment was digested with *Nde*I and *Bam*HI and inserted into pEPP-64S digested with *Nde*I and *Bam*HI to remove the analogous fragment. The PCR-generated region was verified by DNA sequence analysis.

Plasmids were linearized by *Sal*I digestion and transcribed *in vitro* using an Ambion mMessage mMachine SP6 transcription kit. RNA was purified from the transcription mix by making it 0.5 *M* with respect to ammonium acetate and precipitating twice with isopropanol, and the final RNA solution was filtered through a Duropore 0.22-μm filter (Millipore).

Embryo Manipulation

Xenopus eggs were obtained and fertilized as outlined in Jonas *et al.* (1989) and embryos were staged according to Nieuwkoop and Faber (1967). Fertilized eggs at the 1-cell stage were injected into the animal pole with deionized water or RNA in the amount indicated, typically in a volume of 5–10 nl. Injection equipment and methods and culture procedures for injected embryos have been described elsewhere (Jonas *et al.*, 1989). For peptide labeling studies, embryos at stage 7 were injected with 0.5 μCi of [^{35}S]methionine (Amersham, 800 Ci/mmole) according to the procedure of Smith (1986) and incubated for 3 hr. For examination of embryo phosphotyrosine, embryos were incubated in medium containing 1 m*M* Na orthovanadate for 45 min prior to sampling.

For studies involving activin induction, animal caps were cut from stage 8–9 embryos using sharpened watchmaker's forceps and then cultured in 1× MMR (100 m*M* NaCl, 2 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* Hepes, pH 7.4) overnight at 18°C in the presence of 100 μg/ml BSA, at an activin concentration of 3 ng/ml.

Animal caps from EPP-injected embryos used for co-immunoprecipitation of C-cadherin, β-catenin, and α-catenin were dissected at the 32- to 64-cell stage, prior to the formation of lesions. These caps were cultured in 1× MMR containing 0.5 mg/ml BSA until sibling embryos reached stage 9 and had formed obvious lesions.

RNA Isolation and Northern Blotting

RNA was isolated from embryos and animal caps, separated on methylmercury hydroxide gels, blotted, and hybridized with nick-translated DNA probes as described previously (Sargent *et al.*, 1986). RNA integrity was confirmed by ethidium bromide staining of the gel prior to blotting. Probes used were a 632-bp fragment of the Pag cytoplasmic domain (nucleotides 1866 to 2498 of the Pag cDNA; Winning and Sargent, 1994), which will hybridize to EPP RNA, and a *Xenopus* α-actin cDNA (Sargent *et al.*, 1986). Equal RNA loading on Northern blots was confirmed by reprobing blots with cDNA encoding the translation elongation factor EF1-α (Krieg *et al.*, 1989).

Embryo Lysates, Immunoprecipitation, and Immunoblotting

Protein extracts for immunoprecipitations and immunoblotting were made from either embryos or isolated animal caps by homogenization on ice in buffer A (50 m*M* Hepes, pH 7.5, 150 m*M* NaCl, 5 m*M* EDTA, 5 m*M* EGTA, 1% NP-40, 2 m*M* Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml pepstatin, and 10 μg/ml aprotinin. This lysate was then extracted with an equal volume of 1,1,2-trichlorotrifluoroethane (Sigma) and centrifuged at 14,000*g* for 15 min at 4°C to remove yolk proteins. To immunoprecipitate EPP, protein extracts prepared as above were incubated with anti-EGFR monoclonal antibody Ab-1 (Oncogene Sciences) for 30 min on ice. For immunoprecipitation of β-catenin, 2 μl of a *Xenopus* anti-β-catenin antibody (kindly provided by B. Gumbiner and W. Brieher, Memorial Sloan-Kettering Cancer Center) was incubated with protein extracts as

above. To precipitate the immunocomplexes, 20 μl of protein A–sepharose (Pierce) was then added to each reaction and the volume increased to 750 μl with buffer A. The reactions were then rocked for 4 hr at 4°C followed by four washes of 1 ml of buffer A, centrifuging for 5 min between washes. Bound polypeptides were released from the pelleted protein A–Sepharose by resuspending in 1× SDS sample buffer and boiling for 5 min prior to SDS–PAGE. As a control for the detection of phosphotyrosine-containing proteins, extracts of EGF-stimulated A431 cells, supplied by the manufacturers, were used. Phosphorylated EGFR was readily detected in this material by immunoprecipitation and Western blots.

Polypeptides were separated by SDS–PAGE on 7.5% polyacrylamide gels and then fixed and dried under vacuum or electroblotted onto nitrocellulose as described previously (Winning and Browder, 1988). Filters were blocked for 1 hr at room temperature or overnight at 4°C in either 5% BSA in PBST (1× PBS, pH 7.4, 0.1% Tween 20) for immunoblotting with anti-phosphotyrosine (clone 4G10; Upstate Biotechnology Inc, PY-20; Transduction Laboratories) or 5% powdered milk (Carnation) for all other antibodies (anti-β-catenin, anti-α-catenin, and anti XC-cadherin; kindly provided by B. Gumbiner and W. Brieher, Memorial Sloan-Kettering Cancer Center). Blots were incubated with primary antibody for 1 hr at room temperature followed by several washes of PBST. Primary antibodies were detected by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit secondary antibodies (Pierce), at room temperature for 1 hr. Antibody-reactive bands were visualized using an ECL kit (Amersham). Relative molecular masses of polypeptides were determined by comparison to protein standards (New England Biolabs and Pierce).

Embryo Histology

Stage 8.5–9 embryos were fixed in MEMFA (Harland, 1991) and then embedded in JB-4 (Polysciences) and 10-μm sections were cut on a Reichert-Jung Model 2050 microtome. The sections were stained with hematoxylin and eosin and mounted with Polymount (Polysciences). Embryos injected with (β-gal RNA were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 0.2% Nonidet P-40 in 1× PBS. Embryos were stained in 1 mg/ml Xgal, 2 m*M* $MgCl_2$, 5 m*M* $K_4Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$, 1× PBS. Stained embryos were then embedded and sectioned as above.

RESULTS

Biochemical Properties of a Pag–EGFR Chimera in Early Xenopus Embryos

A chimeric receptor clone was created to test the effect on the embryo of ectopic Pag kinase activity. This molecule consists of sequences encoding the extracellular domain (aa 1–621) of the human EGFR joined to sequences encoding the transmembrane and intracellular/catalytic domains of Pag (aa 549–986), as shown in Fig. 1. The resulting chimeric clone, named EPP, encodes a protein of 1084 amino acids with a predicted M_r of 120 kDa. Another chimeric clone was created in parallel that contained two base changes which substitutes alanine for a conserved lysine residue (at position 753) in the ATP-binding domain. This mutation has been shown in other protein tyrosine kinases to abolish kinase activity (Snyder *et al.*, 1985; Ebina *et al.*, 1987; Mitra, 1991). The mutated clone, designated K753A, was used as a control.

The EPP fusion construct behaves as expected when introduced into the cleaving embryo by RNA microinjection. As shown in Fig. 2, injection of EPP RNA results in the synthesis of protein with the appropriate electrophoretic mobility. At RNA doses of 30–100 pg, this protein is phosphorylated on tyrosine residues in response to co-injected RNA encoding transforming growth factor alpha (TGFα), a ligand for EGFR. At RNA doses of 300 pg and above, EPP activates in the absence of ligand. No phosphotyrosine-containing protein was detected in embryos injected with K753A (Fig. 2, lane 7). Clustering of Eph-class receptors by antibodies has been shown to activate receptors (Davis *et al.*, 1994), so it is likely that injection of EPP RNA at these levels produced a sufficient concentration of receptor molecules in the membrane to allow spontaneous dimerization and activation. Most of the experiments reported in this paper were carried out at RNA levels sufficient to elicit autoactivation, alleviating the need for co-injected ligand.

When injected at a dose of 1 ng into 1-cell *Xenopus* embryos, both EPP and K753A RNAs were readily detectable by Northern blot analysis at least until neurula stage (stage 18; Fig. 3A). Protein products of the expected size were detected in total [^{35}S]methionine-labeled protein (Fig. 3B) and in anti-EGFR immunoprecipitates (Fig. 3C) from embryos injected with EPP or K753A RNA, but not from water-injected embryos, indicating that the injected RNAs were being translated, and with approximately equal efficiency. Immunoblotting the EGFR immunoprecipitates with anti-phosphotyrosine antibody (Fig. 3D) demonstrated that EPP was phosphorylated on tyrosine, whereas K753A was not. This confirmed that the K753A mutation abolished kinase activity, as expected, and that there was no other kinase activity in the embryos that will detectably phosphorylate this receptor.

Activated EPP Causes Cell Dissociation in Blastula-Stage Embryos

Embryos injected with RNA encoding EPP developed normally up to early blastula stage. At variable times during blastula stage, but always prior to gastrulation, a subset of the embryos injected with EPP RNA developed conspicuous lesions on the surface of the animal hemisphere (Fig. 4), revealing the unpigmented cells underneath. The appearance of these lesions was invariably accompanied by extensive dissociation in the interior of the embryos, resulting in the occlusion of the blastocoel by nonadherent cells (Fig. 5). An essentially identical effect was obtained in embryos injected with autoactivating levels of RNA encoding full-length Pag protein (data not shown). No dissociation was observed in embryos injected with water or K753A RNA (Figs. 4A and 4C; Table 1). Nor was this phenotype seen in embryos injected with RNA encoding full-length human EGFR at a level eliciting autoactivation (data not shown),

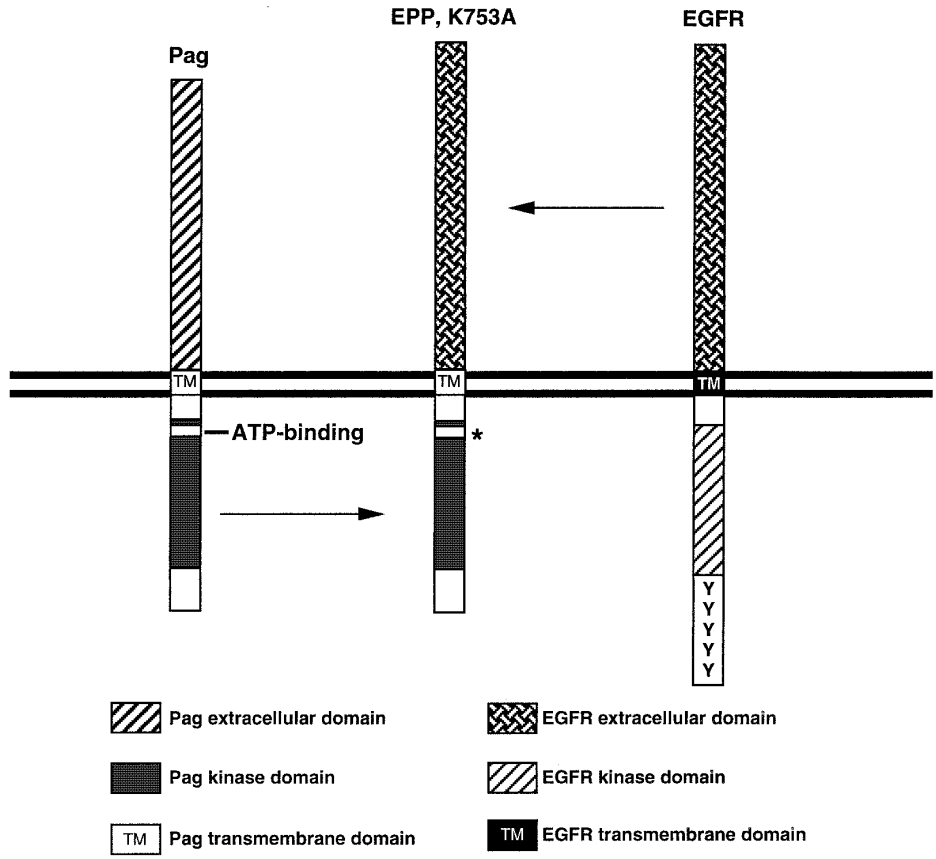

FIG. 1. Diagram of the construction of EPP and K753A. The clones were created by fusing the extracellular domain of human EGFR to the transmembrane and intracellular domains of Pag. For details of the construction, see Materials and Methods. The asterisk shows the approximate location of the mutation introduced into the ATP-binding site of EPP to generate K753A.

indicating that the effect of EPP injection is not due to the EGFR-derived moiety. No difference was observed when the site of injection was targeted to the animal pole versus equatorial injection, except that the external lesion usually was situated near the injection site. Injections into the vegetal pole did not result in obvious dissociation, although such an effect might be difficult to visualize in the large yolk-laden cells located in this region of the cleaving embryo (data not shown). Sections of embryos co-injected with β-galactosidase (β-gal) RNA along with EPP RNA demonstrated that the highest levels of β-gal activity (and therefore presumably the highest levels of β-gal and EPP RNA) were localized to the cells that exhibited the highest levels of disaggregation (Fig. 5D). EPP-induced dissociation does not result in cell death; dissociated blastomeres continue to divide approximately at the same rate as cells in sibling embryos and become readherent after a few hours (data not shown).

In most experiments, the dissociation phenotype was scored by appearance of the external lesions. Table 1 shows the frequency of dissociation as determined in eight experiments. The incidence of dissociation in embryos injected with EPP RNA ranged from 10 to 88%, with a mean of 34%. In all cases, embryos injected with water or with RNA encoding the mutated receptor appeared normal. Therefore, the dissociation phenotype was dependent upon phosphorylation of EPP. As summarized in Table 2, the dissociation effect was observed with EPP RNA doses as low as 30 pg, when co-injected with TGFα mRNA, indicating that the observed loss of cell adhesion does not result from promiscuous EPP activity due to artificially high levels of receptor protein.

Rescue of Pag-Induced Dissociation by K753A and by C-Cadherin Co-injection

If the dissociation resulting from EPP RNA injection were due to kinase activity of the Pag catalytic domain, then co-injection of excess RNA encoding a kinase-negative form of the receptor should rescue embryos via a dominant-negative effect. To test this hypothesis, 4 ng of K753A RNA was co-injected with 1 ng of EPP RNA (Fig. 6). Whereas injection

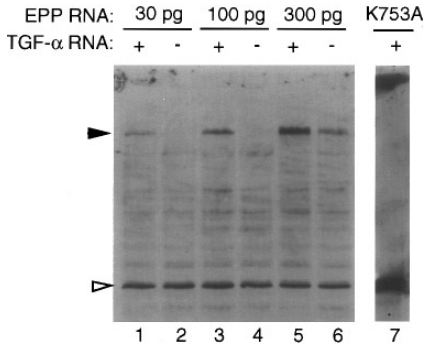

FIG. 2. Co-injection of RNA encoding TGFα activates EPP. Embryos were injected with the indicated amount of EPP RNA (lanes 1–6) either with (+) or without (−) 1 ng of RNA encoding human TGFα. Autophosphorylated EPP (arrowhead) was detected by immunoprecipitating with anti-human EGFR antibody and immunoblotting with anti-phosphotyrosine monoclonal antibody 4G10. Autophosphorylation is ligand dependent with the 100- and 30-pg RNA doses. No tyrosine-phosphorylated proteins are detected in embryo extracts co-injected with 1 ng each of K753A and TGFα RNAs (lane 7). Immunoprecipitating IgG detected by the goat anti-mouse secondary is indicated by an open arrowhead.

of EPP RNA alone (Fig. 6A) resulted in dissociation in 16 of 43 embryos (37%), co-injection of a fourfold excess of mutant RNA (Fig. 6B) reduced the incidence to 1 of 42 embryos (2.3%), and the one external lesion observed was smaller than usual (see arrow in Fig. 6B). EPP-associated tyrosine kinase activity was approximately equal in both samples (Fig. 6C), but most of the phosphotyrosine detected in the co-injected sample was presumably phosphorylated K753A.

Prior to gastrulation, cell–cell adhesion in *Xenopus* embryos is primarily dependent upon the function of two cadherins; C(EP)-cadherin and the less abundant XB(U)-cadherin (Müller *et al.*, 1994; Heasman *et al.*, 1994). It is likely, therefore, that the observed dissociation resulting from Pag activation affects cadherin function in some manner. To test this possibility, embryos were injected with RNAs encoding EPP and TGFα, along with variable amounts of RNA encoding *Xenopus* C-cadherin. As shown in Table 3 and Fig. 7, as little as 1 ng of co-injected C-cadherin RNA can substantially prevent or reverse the dissociation elicited by EPP. In these experiments both the number and size of lesions was reduced in a dose-dependent manner. Western blot analysis showed that the level of C-cadherin in embryos injected with this RNA was increased severalfold over endogenous concentrations and that this did not appreciably lower the accumulation of phosphorylated EPP (Fig. 7E).

Integrity and Phosphorylation of Cadherin–Catenin Complexes

The adhesive function of cadherins depends upon interaction with the cytoskeleton, which is mediated by the cadherin-associated catenins (Schneider *et al.*, 1993, and references therein). To investigate the possibility that the dissociation effect of EPP could be mediated by a disruption of cadherin–catenin complexes in the embryo, co-immunoprecipitation experiments were carried out. Embryos were injected at the 1-cell stage with 1 ng each of EPP (or K753A) and TGFα RNA, and ectodermal cells removed prior to the onset of dissociation. When dissociation was maximal, at mid to late blastula, the explants were extracted and immunoprecipitated with anti-β-catenin, to precipitate the cadherin–catenin complexes. These immunoprecipitates were then analyzed by SDS–PAGE followed by Western blots probed with antibodies specific for C-cadherin, α-catenin, β-catenin, and phosphotyrosine residues. The results, shown in Fig. 8, indicate that EPP activation has no detectable disruptive effect on the interactions between C-cadherin and α- or β-catenin or the level of tyrosine phosphorylation of these proteins.

Inhibition of ras Does Not Prevent EPP-Dependent Dissociation

Transduction of signals originating with receptor tyrosine kinases often requires the activity of the guanine nucleotide-binding protein ras. To test whether signaling by the

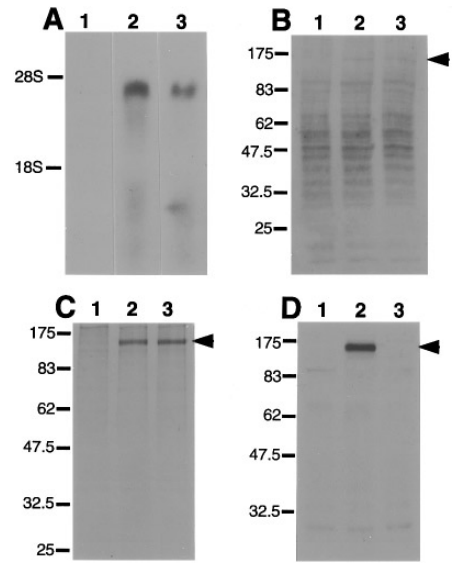

FIG. 3. Expression of injected RNAs in *Xenopus* embryos. In each panel, the order of samples was as follows: lane 1, water-injected; lane 2, injected with 1 ng of EPP RNA; lane 3, injected with 1 ng of K753A RNA. Arrowheads indicate bands of the expected size for EPP and K753A. (A) Northern blot analysis of injected RNAs. (B) Examination of [^{35}S]methionine-labeled protein from whole-cell extract of injected embryos. (C) Immunoprecipitation by antibody against human EGFR of [^{35}S]methionine-labeled embryo lysates. (D) Anti-phosphotyrosine immunoblot of anti-EGFR immunoprecipitates.

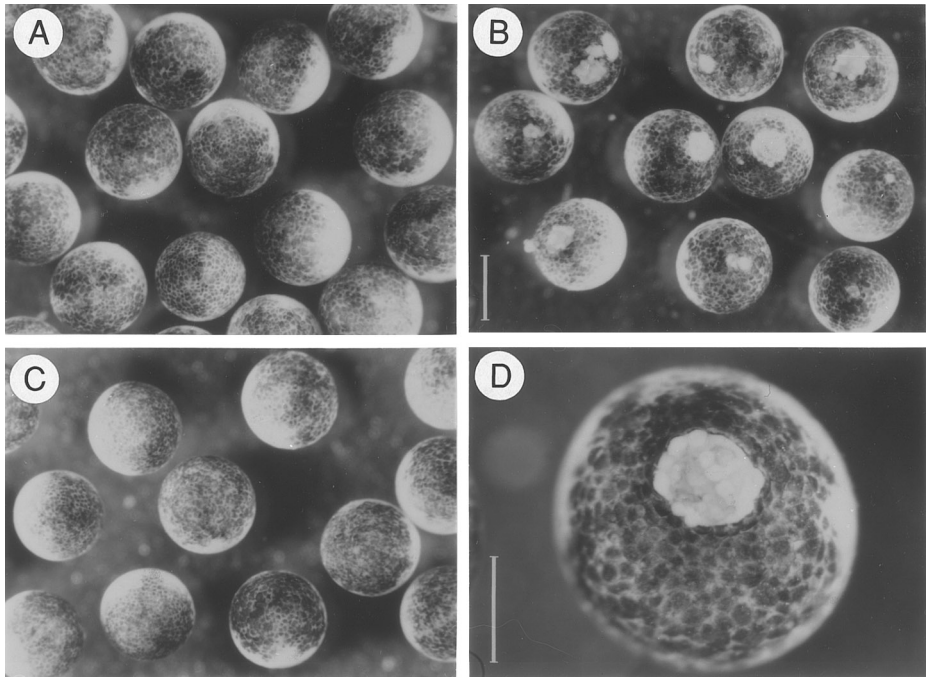

FIG. 4. Effect of RNA injection at 1-cell stage on *Xenopus* embryos at blastula stage. (A) Water-injected embryos. (B) Embryos injected with 1 ng of EPP RNA. (C) Embryos injected with 1 ng of K753A RNA. (D) A higher-magnification view of an embryo injected with EPP RNA. The scale bar in (B) represents 1 mm; the bar in (D) represents 0.5 mm. (A) and (C) are at the same magnification as (B).

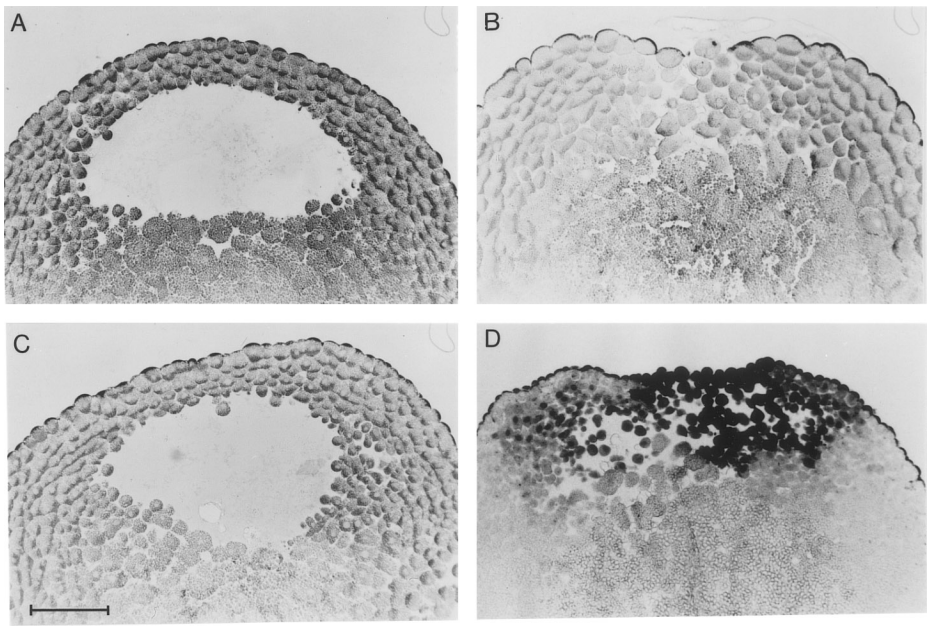

FIG. 5. Sections of injected embryos. The sections in (A), (B), and (C) were stained with hematoxylin/eosin. (A–D) Blastula-stage embryos injected at the 1-cell stage with water (A), 1 ng of EPP RNA (B), 1 ng of K753A RNA (C), or 1 ng EPP RNA and 250 pg of (β-galactosidase RNA, then stained with X-gal after fixation (D). The scale bar in (C) represents 0.2 mm; all panels are at equal magnification.

TABLE 1
Incidence of External Lesion Formation on Injected Embryos

	Injection		
Expt	Water	EPP RNA	EPP-K753A RNA
1	0% (50)	50% (34)	0% (38)
2	0% (59)	38% (43)	0% (45)
3	0% (23)	41% (22)	0% (21)
4	0% (26)	18% (39)	0% (20)
5	0% (50)	10% (50)	0% (48)
6	0% (58)	28% (58)	0% (61)
7	0% (45)	34% (38)	0% (41)
8	0% (36)	32% (38)	0% (46)
Total	0% (337)	35% (332)	0% (320)

Note. Percentages indicate the fraction of embryos exhibiting surface lesions. Numbers in parentheses are total embryos examined in each sample.

Pag catalytic domain exhibits such a dependence, we co-injected 0.5 ng of RNA encoding a dominant-negative ras (S17N ras; Whitman and Melton, 1992) along with EPP RNA. Effectiveness of the dominant-negative ras was assayed by treating animal caps from S17N ras-injected embryos with activin and measuring the induction of α-actin, a marker for mesoderm induction (Sargent *et al.*, 1986). Mesoderm induction by activin requires ras activity (Whitman and Melton, 1992), so inhibition of α-actin mRNA accumulation, a gauge of mesoderm induction, serves as a relative measure of ras inactivation by S17N ras. As shown in Fig. 9, injection of S17N ras reduced α-actin levels in activin-treated animal caps six- to sevenfold compared to those in uninjected, activin-treated caps. The effect of S17N ras and EPP RNA co-injection is shown in Table 4. Embryos injected with S17N ras RNA alone appeared to develop normally to late blastula stage. As stated above, these embryos failed to gastrulate. Co-injection of S17N ras with EPP was observed to have little effect on embryo dissociation. Embryos co-injected with S17N ras and K753A were indistinguishable from embryos injected with either RNA alone.

A similar result was obtained when embryos were examined internally at stage 9. Fourteen of fifteen embryos injected with EPP RNA showed extensive internal cell dissociation. The same number of embryos co-injected with EPP and S17N ras RNA showed internal dissociation. Control embryos, injected with water or K753A RNA alone, appeared normal. These results indicate that the EPP signal resulting in loss of cell adhesion is not dependent on activation of the ras pathway.

DISCUSSION

Eph-class receptor tyrosine kinases originally attracted interest due to the localized expression patterns of many members of this family, particularly within the developing nervous system. More recently, Eph-class RTKs and ligands have been implicated in mediating repulsive signals in axon fasciculation and retinotectal axon targeting (Winslow *et al.*, 1995; Drescher *et al.*, 1995). Also, experiments with dominant negative molecules have shown that XSek-1, which is essentially identical to Pag, can disrupt rhombomere boundaries in zebrafish and *Xenopus* (Xu *et al.*, 1995) and can also lead to alterations in forebrain patterning in the zebrafish (Xu *et al.*, 1996). The molecular and cellular basis for these developmental functions is unknown. However, cell motility and adhesion are likely to be involved. In this paper we show that signaling through one Eph-class receptor, Pag, has a dramatic inhibitory effect on cell adhesion in cleaving *Xenopus* embryos, possibly by modulating cadherin function.

The majority of our experiments used an amount of EPP RNA which resulted in a level of chimeric receptor high enough to promote spontaneous dimerization and autophosphorylation. However, when ligand (TGFα) was supplied, injection of as little as 30 pg of EPP RNA resulted in embryonic dissociation. Pag mRNA falls into the rare class of mRNAs in whole embryos, corresponding to approximately 1–10 pg of mRNA per embryo. However, the expression of the Pag gene is highly localized, so in tissues where it is transcribed, the endogenous Pag mRNA con-

TABLE 2
Incidence of External Lesion Formation in Embryos Injected with Various Doses of EPP RNA and with or without Co-injection of TGFα RNA

		EPP RNA			
Expt.	TGFα RNA	300 pg	100 pg	30 pg	0 pg
1	0 ng	17% (42)	6% (47)	0% (34)	0% (50)
	1 ng	44% (45)	24% (49)	6% (51)	0% (50)
2	0 ng	24% (46)	0% (50)	0% (49)	0% (61)
	1 ng	48% (46)	21% (47)	16% (44)	0% (50)

Note. Percentages indicate the fraction of embryos exhibiting surface lesions. Numbers in parentheses are total embryos examined in each sample.

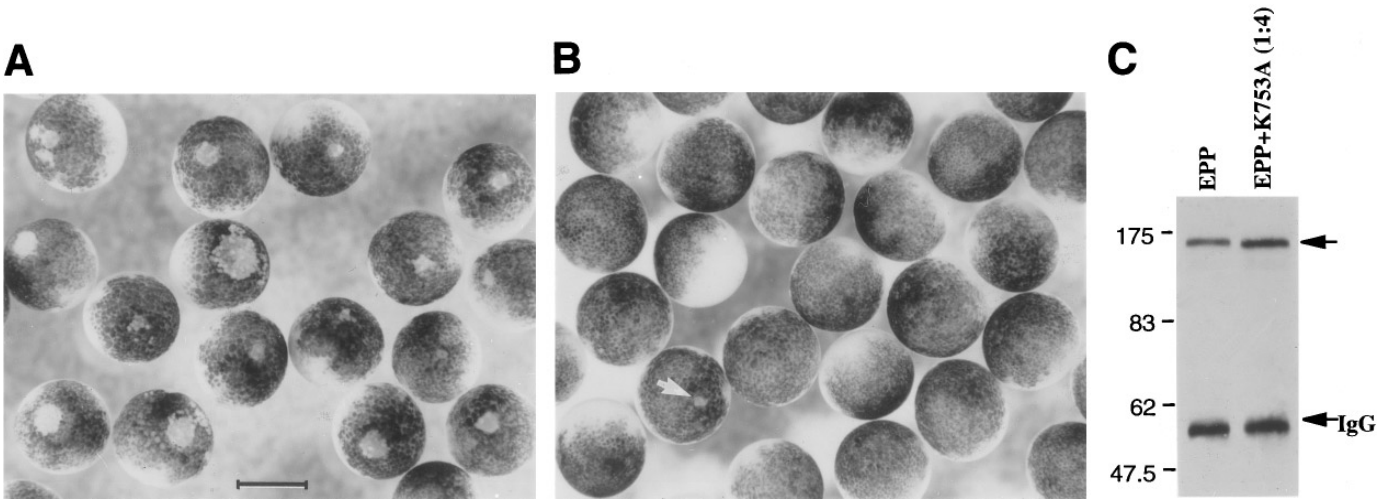

FIG. 6. Rescue of EPP-injected embryos by co-injection of K753A RNA. (A) Blastula-stage embryos injected at the 1-cell stage with 1 ng of EPP RNA. Bar, 1 mm. (B) Blastula-stage embryos injected at the 1-cell stage with 1 ng of EPP RNA and 4 ng of K753A RNA. The arrow indicates the single embryo in this sample that exhibited a lesion. (C) Anti-phosphotyrosine immunoblot of anti-EGFR immunoprecipitates of the embryos in (A) and (B). The arrow represents EPP-associated phosphorylation.

centration should be comparable to the levels of experimentally injected EPP sequences. Therefore, embryonic dissociation elicited by EPP takes place at physiologically relevant receptor levels.

The pathway through which Pag activation achieves embryonic dissociation remains unknown. However, cell adhesion in early frog development depends primarily on the function of adherens junctions, which comprise the maternal cadherins C/EP and U/XB (Müller *et al.*, 1994) and associated catenins. Therefore, the function of these proteins is likely to be affected in some way by Pag. In chick embryonic fibroblasts transformed with Rous sarcoma virus, cadherin-dependent cell adhesion is disrupted. This is accompanied by tyrosine phosphorylation of the cadherin–catenin complex by viral src protein, but the integrity of cadherin–catenin complexes does not appear to be disturbed (Hamaguchi *et al.*, 1993). We do not detect any tyrosine phosphorylation of C-cadherin or α- or β-catenin, or detectable disruption of cadherin–catenin complexes in EPP-injected embryos (Fig. 8). Nevertheless, the observation that increasing the level of C-cadherin prevents or reverses the dissociation caused by EPP supports the conclusion that this or another cadherin is the ultimate target of Pag action.

Several models can be envisioned whereby Pag or other Eph-class RTKs could elicit changes in cell adhesion. One possibility is that tyrosine phosphorylation of Pag (or EPP) leads to a direct interaction between the receptor and cadherin or to recruitment of other proteins to the adherens junctions. Either of these events could in turn interfere with cell adhesion. Attempts to demonstrate stable cadherin–receptor complex formation by co-immunoprecipitation assays have not been successful (R. S. Winning, unpublished), but the possibility of labile or transient interactions has not been excluded.

If signaling through Pag can reduce cadherin-dependent cell adhesion, what significance might this have in the developing embryo? In *Xenopus*, Pag mRNA first appears at the early gastrula stage (Winning and Sargent, 1994). Western blot analysis indicates there is not a significant delay in the translation of this mRNA (J. B. Scales, unpublished), so Pag protein is probably present throughout gastrulation. Experiments in which dominant-negative C-cadherin RNA was injected into *Xenopus* embryos indicate that this adhesion molecule is important in the correct execution of gastrulation movements (Lee and Gumbiner, 1995). Based on these results, and the findings presented in this paper on the ability of Pag to influence cell adhesion, it is reasonable to hypothesize a role for this receptor in the gastrulation process.

TABLE 3

Rescue of EPP-Induced Lesions by Co-injection of C-cadherin RNA

	C-cadherin RNA (ng)			
	0	1	2	4
Expt. 1	64% (121)	30% (80)	15% (81)	10% (82)
Expt. 2	88% (108)	8% (74)	5% (77)	3% (80)

Note. Embryos injected at the 1- or 2-cell stage with 1 ng EPP RNA, 1 ng TGFα RNA, and the indicated amount of C-cadherin RNA. Percentages indicate the fraction of embryos exhibiting surface lesions. Numbers in parentheses are total embryos examined in each sample.

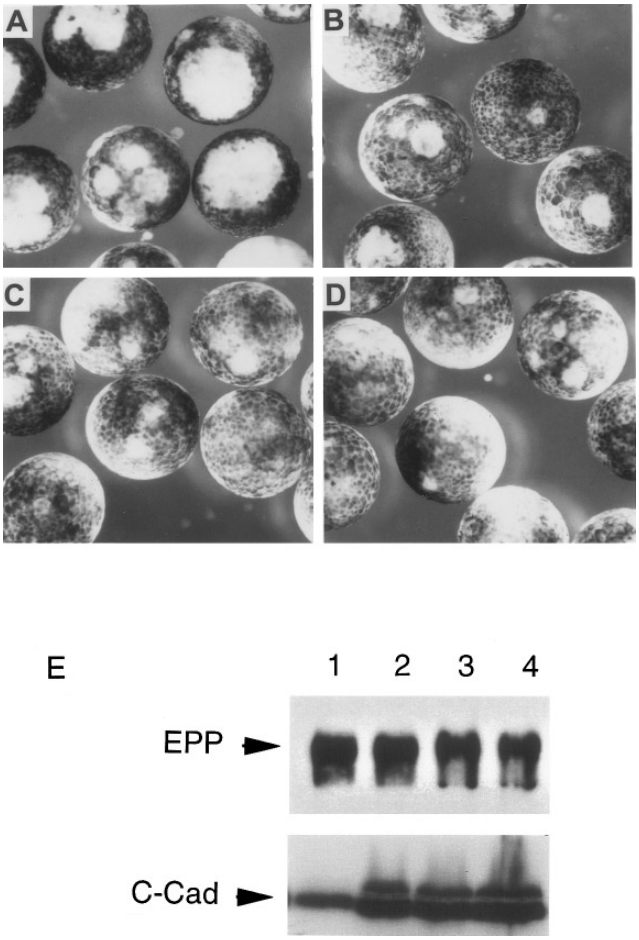

FIG. 7. Rescue of EPP-injected embryos by co-injection of C-cadherin RNA. (A) Blastula-stage embryos injected at the 1-cell stage with 1 ng each of EPP and TGFα RNA, showing extensive dissociation. (B–D) Blastula-stage embryos co-injected at the 1-cell stage with 1 ng each of EPP and TGFα and either 1 ng (B), 2 ng (C), or 4 ng (D) of C-cadherin RNA. Note that these panels show embryos selected for the appearance of external dissociation lesions. The frequency of lesions is markedly decreased in the embryos co-injected with C-cadherin RNA (see Table 3). (E) Proteins isolated from embryos injected as above either immunoprecipitated with anti-EGFR and immunoblotted with anti-phosphotyrosine (upper panel) or immunoblotted directly with anti-C-cadherin (lower panel). Lanes 1–4 correspond to embryos injected as in A–D, respectively. Co-injection of these RNAs does not result in any significant reduction in the activation of EPP.

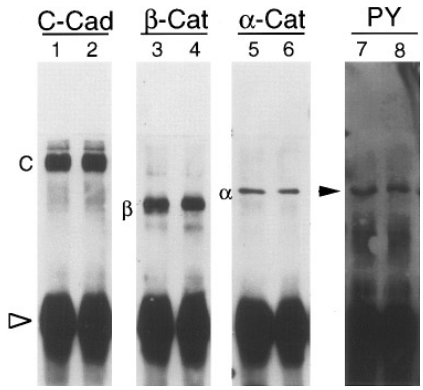

FIG. 8. Immunoprecipitation and immunoblot analysis of injected embryos. Lysates from ectoderm dissected from embryos injected with either EPP (lanes 1, 3, 5, and 7) or K753A (lanes 2, 4, 6, and 8) were immunoprecipitated with anti-β-catenin antibody (lanes 1–6), followed by immunoblot analysis using antibodies against *Xenopus* C-cadherin (lanes 1 and 2), *Xenopus* β-catenin (lanes 3 and 4), and *Xenopus* α-catenin (lanes 5 and 6). Embryo lysates immunoprecipitated with anti-*Xenopus* C-cadherin antibody were immunoblotted with anti-phosphotyrosine (lanes 7 and 8). The immunoblotting antibody is indicated above each set of lanes and the corresponding protein band indicated by a C, β, or α adjacent to its position. The only major peptide detected by anti-phosphotyrosine is indicated by an arrowhead. In no case is there any discernible difference between embryos injected with EPP compared to K753A in the amount or mobility of the proteins detected. Detection of the immunoprecipitating IgG by the secondary antibody is indicated by an open arrowhead.

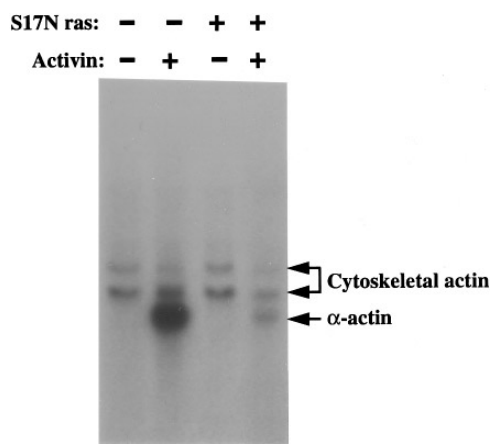

FIG. 9. Inhibition of ras activity in animal caps from embryos injected with S17N ras RNA. Embryos were injected at the 1-cell stage with water (−) or RNA encoding S17N ras (+). Animal caps from injected embryos were left untreated (−) or treated (+) with activin, and α-actin mRNA levels were determined by Northern blot analysis. Arrows differentiate between the ubiquitous cytoskeletal actin mRNAs and the mesoderm-specific α-actin mRNA.

It is conceivable that the establishment or maintenance of rhombomere boundaries (Xu *et al.*, 1995; 1996) could depend on regulation of cadherin function by XSek-1/Pag. If cells expressing Pag (but not expressing Pag ligands) invaded a territory in which cells expressed an activating ligand, one would predict from our results that the invading cell would down-regulate cadherin function due to activa-

TABLE 4

Incidence of External Lesion Formation and Internal Dissociation in Embryos Injected with EPP and S17N ras RNAs

Expt.	Injection	
	EPP	EPP + S17N ras
1	88% (43)	71% (41)
2	34% (38)	77% (43)
3	10% (50)	31% (45)
Internal dissociation (Expt 3)	93% (15)	93% (15)

Note. Percentages indicate the fraction of embryos exhibiting surface lesions or internal dissociation. Numbers in parentheses are total embryos examined in each sample.

tion of the receptor. This could lead to exclusion of the cell from adherent neighbors, until it departed the region presenting the Pag ligand, at which time the block to cadherin function would be released. Such a model would be consistent with the cell mixing patterns observed in chick rhombomere transplantation experiments (Guthrie *et al.*, 1993).

One could also speculate that cadherin modulation by Eph-class RTKs might play a role in the mechanism by which these receptors influence retinotectal targeting. The repulsive signal mediated by the Eph-class ligand RAGS appears to result in growth cone collapse (Drescher *et al.*, 1995), an incompletely understood process that probably entails alterations in the cytoskeleton and the cell adhesion properties of affected axons (Keynes and Cook, 1995; Tanaka and Sabry, 1995). It is possible that activation of Mek-4 or another Eph-class RTK could result in down-regulation of cell adhesion in the growth cone, by a mechanism similar to the loss of adhesion we have observed with *Xenopus* embryos injected with EPP.

It is highly probable that morphogenetic movement of cells requires tissue-specific cell–cell adhesion that can be modulated in response to developmental cues. The results presented in this paper suggest that interactions between the Eph family of receptor tyrosine kinases and the cadherin family of adhesion molecules, both of which exhibit localized expression during development, could be the basis of such a regulatory mechanism.

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REFERENCES

Bartley, T. D., Hunt, R. W., Welsher, A. A., Boyle, W. J., Parker, V. P., Lindberg, R. A., Lu, H. S., Colombero, H. M., Elliot, R. L., Guthrie, B. A., Holst, P. L., Skrine, J. D., Toso, R. J., Zhang, M., Fernandez, E., Trail, G., Varnum, B., Yarden, Y., Hunter, T., and Fox, G. M. (1994). B61 is a ligand for the Eck receptor protein-tyrosine kinase. *Nature* **368,** 558–560.

Beckmann, M. P., Cerretti, D. P., Baum, P., Vanden Bos, T., James, L., Farrah, T., Kozlosky, C., Holligsworth, T., Shilling, H., Maraskovsky, E., Fletcher, F. A., Lhotàk, V., Pawson, T., and Lyman, S. D. (1994). Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors. *EMBO J.* **13,** 3757–3762.

Brambilla, R., Schnapp, A., Casagranda, F., Labrador, J. P., Bergemann, A. D., Flanagan, J. G., Pasquale, E. B., and Klein, R. (1995). Membrane-bound LERK2 ligand can signal through three different Eph-related receptor tyrosine kinases. *EMBO J.* **13,** 3116–3126.

Cheng, H.-J., and Flanagan, J. G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell* **79,** 157–168.

Cheng, H.-J., Nakamoto, M., Bergemann, A. D., and Flanagan, J. G. (1995). Complementary gradients if expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* **82,** 371–381.

Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotàk, V., Pawson, T., Goldfarb, M., and Yancopoulos, G. D. (1994). Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* **266,** 816–819.

Drescher, U., Kremoser, C., Handwerker, C., Löschinger, J., Noda, M., and Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for the eph receptor tyrosine kinases. *Cell* **82,** 359–370.

Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A., and Rutter, W. J. (1987). Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. *Proc. Natl. Acad. Sci. USA* **84,** 704–708.

Fox, G. M., Holst, P. L., Chute, H. T., Lindberg, R. A., Janssen, A. M., Basu, R., and Welcher, A. A. (1995). cDNA cloning and tissue distribution of five human EPH-like receptor protein-tyrosine kinases. *Oncogene* **10,** 897–905.

Gilardi-Hebenstreit, P., Nieto, M. A., Frain, M., Mattei, M. G., Chestier, A., Wilkinson, D. G., and Charnay, P. (1992). An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the developing mouse hindbrain. *Oncogene* **7,** 2499–2506.

Guthrie, S., Prince, V., and Lumsden, A. (1993). Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts. *Development* **118,** 527–538.

Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M., and Nagai, Y. (1993). $p60^{v\text{-}src}$ causes tyrosine phosphorylation and inactivation of the N-cadherin–catenin cell adhesion system. *EMBO J.* **12,** 307–314.

Harland, R. M. (1991). In situ hybridization: An improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol.* **36,** 685–695.

Heasman, J., Ginsberg, D., Geiger, B., Goldstone, K., Pratt, T., Yoshida-Noro, C., and Wylie, C. (1994). A functional test for mater-

nally inherited cadherin in *Xenopus* shows its importance in cell adhesion at the blastula stage. *Development* **120,** 49–57.

Jonas, E. A., Snape, A. M., and Sargent, T. D. (1989). Transcriptional regulation of a *Xenopus* embryonic epidermal keratin gene. *Development* **106,** 309–405.

Keynes, R., and Cook, G. M. W. (1995). Axon guidance molecules. *Cell* **83,** 161–169.

Kozlosky, C. J., Maraskovsky, E., McGrew, J. T., VandenBos, T., Teepe, M., Lyman, S. D., Srinivasan, S., Fletcher, F. A., Gayle, R. B. III, Cerretti, D. P., and Beckmann, M. P. (1995). Ligands for the receptor tyrosine kinases hek and elk: isolation of cDNAs encoding a family of proteins. *Oncogene* **10,** 299–306.

Krieg, P. A., Varnum, S. M., Wormington, W. M., and Melton, D. A. (1989). The mRNA encoding elongation factor 1-alpha (EF-1 alpha) is a major transcript at the midblastula transition in *Xenopus. Dev. Biol.* **133,** 93–100.

Lee, C. H., and Gumbiner, B. M. (1995). Disruption of gastrulation movements in *Xenopus* by a dominant-negative mutant for C-cadherin. *Dev. Biol.* **171,** 363–373.

Lhotàk, V., and Pawson, T. (1993). Biological and biochemical activities of a chimeric epidermal growth factor-Elk receptor tyrosine kinase. *Mol. Cell. Biol.* **13,** 7071–7079.

Lonardo, F., Di Marco, E., King, C. R., Pierce, J. H., Segatto, O., Aaronson, S. A., and Di Fiore, P. P. (1990). The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. *New Biol.* **2,** 992–1003.

Mitra, G. (1991). Mutational analysis of conserved residues in the tyrosine kinase domain of the human trk oncogene. *Oncogene* **6,** 2237–2241.

Müller, H.-A., Kühl, M., Finnemann, S., Schneider, S., van der Poel, S. Z., Hausen, P., and Wedlich, D. (1994). *Xenopus* cadherins: The maternal pool comprises distinguishable members of the family. *Mech. Dev.* **47,** 213–223.

Nieuwkoop, P. D., and Faber, J. (1967). ''Normal Table of *Xenopus laevis* (Daudin).'' North-Holland, Amsterdam.

Sajjadi, F. G., and Pasquale, E. B. (1993). Five novel avian eph-related tyrosine kinases are differentially expressed. *Oncogene* **8,** 1807–1813.

Sargent, T. D., Jamrich, M., and Dawid, I. B. (1986). Cell interactions and the control of gene activity during early development of *Xenopus laevis. Dev. Biol.* **114,** 238–246.

Scales, J. B., Winning, R. S., Renaud, C. S., Shea, L. J., and Sargent, T. D. (1995). Novel members of the Eph receptor tyrosine kinase subfamily expressed during Xenopus development. *Oncogene* **11,** 1745–1752.

Schneider, S., Herreknecht, K., Butz, S., Kemler, R., and Hausen, P. (1993). Catenins in Xenopus embryogenesis and their relation to the cadherin-mediated cell-cell adhesion system. *Development* **118,** 629–640.

Shao, H., Lou, L., Panday, A., Pasquale, E. B., and Dixit, V. M. (1994). cDNA cloning and characterization of a ligand for the Cek5 receptor protein-tyrosine kinase. *J. Biol. Chem.* **269,** 26606–26609.

Smith, R. C. (1986). Protein synthesis and messenger RNA levels along the animal-vegetal axis during Xenopus development. *J. Embryol. Exp. Morphol.* **95,** 15–35.

Snyder, M. A., Bishop, J. M., McGrath, J. P., and Levinson, A. D. (1985). A mutation at the ATP-binding site of pp60v-src abolishes kinase activity, transformation, and tumorigenicity. *Mol. Cell. Biol.* **5,** 1772–1779.

Tanaka, E., and Sabry, J. (1995). Making the connection: cytoskeletal rearrangements during growth cone guidance. *Cell* **83,** 171–176.

Tuzi, N. L., and Gulick, W. J. (1994). Eph, the largest known family of putative growth factor receptors. *Br. J. Cancer* **69,** 417–421.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* **309,** 418–425.

Whitman, M., and Melton, D. (1992). Involvement of p21 ras in Xenopus mesoderm induction. *Nature* **357,** 252–255.

Winning, R. S., and Browder, L. W. (1988). Changes in heat shock protein synthesis and hsp70 gene transcription during erythropoiesis in *Xenopus laevis. Dev. Biol.* **128,** 111–120.

Winning, R. S., and Sargent, T. D. (1994). Pagliaccio, a member of the Eph family of receptor tyrosine kinase genes, has localized expression in a subset of neural crest and neural tissues in *Xenopus laevis* embryos. *Mech. Dev.* **46,** 219–229.

Xu, Q., Alldus, G., Holder, N., and Wilkinson, D. G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the *Xenopus* and zebrafish hindbrain. *Development* **121,** 4005–4016.

Xu, Q., Alldus, G., Macdonald, R., Wilkinson, D. G., and Holder, N. (1996). Function of the Eph-related kinase rtk1 in patterning of the zebrafish forebrain. *Nature* **381,** 319–322.

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